

Optimization and Characterization of Tyrosinases from Multi-enzyme Producing *Fusarium solani* and *Fumago* sp.

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Abstract

Tyrosinase is a copper-containing metalloprotein that catalyzes the oxidation of tyrosine, in particular, L-DOPA to L-Dopaquinone, which are precursors of brown pigments in some wounded eukaryotic tissues. The present study focused on screening, production and characterization of tyrosinase from multi-enzyme producing *Fusarium solani* B1 and *Fumago* sp. A total of 25 strains were isolated from rotting wood samples and screened for hydrolytic and oxidative multi-enzyme potentials using different polymeric substrates. The two most consistent strains: *Fusarium solani* B1 and *Fumago* sp. B13 were further evaluated for tyrosinase production. Some media cultural parameters and physiological conditions were optimized in order to maximize tyrosinase production. Incubation of *Fumago* sp. B13 and *Fusarium solani* B1 for 96 and 144 h in medium containing 2 % and 0.2 % ratios of Glucose and NaNO₃ with pH 6 and 7, respectively, was most suitable for tyrosinase production. Characterization of the partially purified tyrosinase from *Fumago* sp. B13 and *Fusarium solani* B1 exhibited optimal activities at pH 6-7, 30 °C, and 1 mM Cu²⁺, respectively, thereby suggesting their potentials for novel biotechnological applications.

Keywords

Fusarium solani B1, *Fumago* sp B13, multi-enzyme, tyrosinase, characterization

1 Introduction

With the advent of biotechnology, there has been a growing interest and demand for enzymes with novel properties. Considerable efforts are being directed towards the selection of microorganisms producing enzymes with enhanced catalytic properties and tolerance to extreme conditions characteristic of industrial processes, such as temperature, salts, pH and many others. The potentials of using fungi as biotechnological sources of industrially relevant enzymes have stimulated interest in the exploration of their extracellular enzyme activity. Multi-enzymes could be produced through their induction on exposure to certain cultural conditions [1], through engineered microbes with multi-functions [2], through mixed culture of microbes [3], or ultimately, through directed evolution of enzymes [4]. High yielding strains, optimal fermentation conditions and efficient separation technologies have been anticipated to reduce the cost of producing many enzymes. Notwithstanding, pure enzyme

extracts might still contain trivial amounts of a variety of accessory enzymes, which may be responsible for their multicatalytic potentials.

Tyrosinase is a copper containing enzyme present in both plant and animal tissues that catalyzes the production of melanin and other pigments from tyrosine by oxidation. A typical illustration is the blackening of a peeled or sliced tuber crop, during exposure to air [5]. From a mechanistic perspective, tyrosinases oxidize their substrates through hydroxylation of monophenol to *o*-diphenol (monophenolase or cresolase activity) and the oxidation of diphenol to *o*-quinones (diphenolase or catecholase activity), both using molecular oxygen, followed by a series of non-enzymatic steps, which may result in the formation of melanin [6]. These reactions may be responsible for the undesired enzymatic browning of fruits and vegetables, due to tissue injuries that may occur during senescence, harvest or postharvest handling. However, besides undesired

browning, tyrosinases have been extolled for their role in the much desired organoleptic features of raisins, cocoa and fermented tea leaves among many others. In fungi, this phenomenon is associated with the differentiation of reproductive organs and spore formation, virulence of pathogenic fungi, and tissue protection after injury [7]. They achieve these tasks through the synthesis of melanin as a mechanism of defense against ultraviolet radiation, free radicals, gamma rays and extreme environmental conditions, as well as its contribution to the cell wall against hydrolytic enzymes, providing cell lysis.

Although tyrosinases have been scarcely considered, compared to its oxidative *confrère*, in the treatment of wastewater micropollutants, its biotechnological relevance in pharmaceuticals, nutraceuticals, and cosmeceuticals is renowned [8, 9]. Interestingly, tyrosinases are being considered as a constituent of a functional amalgam-biosensor for the detection of inconsequential levels of volatile phenolics [10], thus highlighting its accuracy and sensitivity. Mushroom tyrosinases have attracted much biochemical interest due to the potentials already stated, and it has encouraged the exploratory search for novel fungal sources. A novel tyrosinase producing actinomycete strain has been isolated from Sahara soil [11]. Similarly, a study has been earlier conducted on two basidiomycetes, *Agaricus bisporus* and *Pleurotus ostreatus*, which had their tyrosinases purified and characterized [9]. However, to our knowledge, this is the first account of tyrosinase production from *Fumago* sp. and its characterization.

2 Materials and methods

2.1 Collection and isolation of microorganisms

Seven chunks of rotting wood samples and eight mushroom samples, growing on rotting logs of wood were collected and rinsed in 1 % hypochlorite solution (to reduce microbial contamination) for 10 min then thoroughly rinsed with sterile water. These samples (5 mm²) were centrally located on Petri dishes containing sterilized and cooled potato dextrose agar (LAB-M) to which 100 µg mL⁻¹ of streptomycin was added to inhibit bacterial growth. Plates were incubated at room temperature for 120 h. The growths which occurred on the plates as fungal mycelia were subcultured by aseptically transferring 8 mm mycelia plugs successively onto fresh PDA plates with the aid of a sterile cork borer, until pure cultures were observed and these were maintained on potato dextrose agar slants at 4 °C.

2.2 Biocatalytic activity of fungal strains

The fungi isolated were qualitatively screened for catalytic enzymes such as amylases, cellulases, tannases, pectinases, proteases, mannases and tyrosinases. Agar plugs (8 mm) from 7 day old fungal cultures were aseptically and centrally inoculated on Petri plates containing about 15 mL of different screening media, and were analyzed as described below. Amylase was determined on soluble starch agar medium comprising (g/L) starch: 20; yeast extract: 0.5; KH₂PO₄: 10 ; (NH₄)₂SO₄: 10.5; MgSO₄·7H₂O: 0.3; CaCl₂: 0.5; to which 1 mL of a cocktail of elements (g/L): FeSO₄·7H₂O: 0.004; MnSO₄·7H₂O: 0.004 and ZnSO₄·H₂O: 0.004 was added. The plates were incubated and observed for 72 h after which they were flooded with Gram's Iodine. A clear hydrolytic zone around the cultures on the starch plates against a blue/black background was taken as positive, while a negative experiment would not reveal any zone around the inoculated culture.

Cellulase was determined on a medium described by Islam and Roy [12] with slight modifications, which entailed the replacement of 2 g gelatin with 0.5 g yeast extract. Tannic acid agar medium (TAA) as described by Pinto et al. [13] was used for screening of tannase activity. Pectinase was determined on 1 % citrus pectin supplemented Czapek mineral salt screening agar medium containing (g/L): NaNO₃: 2; K₂HPO₄: 1.0; MgSO₄·7H₂O: 0.5; KCl: 0.5; Peptone: 2; Agar: 20; pH 6.5. Protease activity was screened for on a skim milk medium described by Polley and Ghosh [14]. Mannanase activity was assayed on a basal salt medium containing (g/L): NaNO₃: 2.0; K₂HPO₄: 1.0; MgSO₄·7H₂O: 0.5; KCl: 0.5; Peptone: 2.0; Agar: 20; pH 6.5 supplemented with 1 % gum acacia. Tyrosinase was screened for on a medium described by Raval et al. [15]. The tyrosine screening medium contained (g/L): Peptone: 5; Yeast Extract: 3; L-tyrosine: 5; Agar: 20; pH 7. All the plates were incubated at 28 °C for 72 h and thereafter stained with iodine, except for protease plate, which exhibited a clear zone post-incubation, as well as tannase and tyrosinase screening plates, which were only observed for color changes. The appearance of brown halos around fungal growth was indicative of hydrolytic or oxidative activities of the respective enzymes.

2.3 Fungal selection and identification

The isolates were identified macroscopically and microscopically using the slide agar method [16] after staining with lactophenol cotton blue stain. Simplified key to some selected common genera [17] was used for identification of isolates to the genus level.

2.4 Production of tyrosinase

Tyrosinase was produced following the method of Roy et al. [18]. Erlenmeyer flasks (250 mL) containing 100 mL of tyrosinase production broth (g/L): Starch; 25, Glucose; 10, Yeast extract; 2, CaCO_3 ; 3, and 1 mL of a trace salt solution containing (1 % of FeSO_4 , CuSO_4 , ZnSO_4 , MnCl_2), were autoclaved at 121°C for 15 min. After cooling, 10 mL 0.1 % of tyrosine was added to the medium and each flask was inoculated with five agar plugs (5 mm diameter) from 72 h old fungal cultures and incubated at room temperature for seven days. After the days of incubation, the extracellular enzyme from each fungal isolate was obtained by centrifuging the biomass at 8000 rpm, 4 °C for 15 min and the supernatant was assayed for tyrosinase activity.

2.5 Determination of tyrosinase activity

The continuous spectrophotometric rate determination method of Naraoka et al. [19] was employed, with modifications. Reaction cocktails containing (0.9 mL of deionized water, 1 mL of 0.5 M sodium phosphate buffer at pH 6.5 and 1 mL of 0.2mM tyrosine solution) were prepared and mixed at 25 °C. The mixture was shaken with a vortexer for 3 to 5 min after which 0.1 mL of the enzyme sample was added to the cocktail. One international unit (IU) of enzyme activity was described as the amount that catalyzes the transformation of 1 μmole of substrate to product per min at pH 6.5 at 25 °C in a 3 mL reaction mix, and was calculated as follows, using the molar absorption coefficient of dopachrome ($3600 \text{ M}^{-1} \text{ cm}^{-1}$):

$$IU = \frac{\Delta A}{\Delta t} \times 10^6 \mu\text{m} \times V_f (L) / \varepsilon \times M \quad (1)$$

where;

ε = molar absorption coefficient

V_f = final volume in the cuvette (L)

A = absorbance

t = time (min).

2.6 Optimization of fermentation conditions for tyrosinase production and time course study

Fermentation experiments were conducted in 250 mL Erlenmeyer flasks containing 100 mL modified basal salt medium. Tyrosinase production was optimized by a traditional ‘one factor at a time’ (OFAT) approach, which entailed varying pH (3-9) using phosphate buffers (pH 4-7) and tris buffer (pH 8 and 9). Carbon sources (1 %) (maltose, lactose, fructose, sucrose) were varied, using glucose as control, as well as 0.2 % nitrogenous

sources (peptone, ammonium sulphate, urea and sodium nitrate) where yeast extract served as the control. Time course of tyrosinase production was determined by the extraction of aliquots of fermented culture broth at successive periods of incubation for analysis.

2.7 Partial purification of tyrosinase from the crude extract

A hundred millilitre (100 mL) supernatant from a fungal culture broth was obtained through centrifugation at $8000 \times g$ for 15 min and concentrated with ammonium sulphate (40 % saturation). After overnight storage at 4 °C, the ammonium sulphate precipitated protein was centrifuged at 10000 rpm, 4 °C for 20 min. The precipitate (10 mL) was resuspended in 90 mL of 0.2 M phosphate buffer (pH 7.0) with continuous stirring under refrigeration and was then checked for enzymatic activity as well as total protein content. Thereafter, dissolved precipitates were transferred into a preconditioned dialysis bag and sealed at both ends. The dialysis bag with its content was suspended overnight at 4 °C in a glass beaker containing 0.2 M phosphate buffer (pH 7.0) with stirring. The dialysate was checked for tyrosinase activity [18] and the total protein content measured following the Bradford method of 1976 [20].

2.8 Tyrosinase characterization

Temperature effects on tyrosinase activity was determined at different temperature ranging from 30 °C-70 °C by pre-incubating the purified extracellular enzyme at the stated temperature ranges for 15 min in a constant temperature water bath. After this, the residual tyrosinase activity was determined [21] as earlier described in Section 2.5.

The effect of pH modifications on tyrosinase activity was determined by preincubating equal volumes of the enzyme and phosphate buffer (pH 5.0-7.0) and tris buffer (8.0 and 9.0) at 28 °C for 15 min. The activity in the reaction mixtures was determined under the stated assay conditions. The effect of 1 mM metal ions (Hg^{2+} , Ca^{2+} , Mn^{2+} , Mg^{2+} and Cu^{2+}) from chloride salts of the metals on tyrosinase activity was determined. The enzyme was incubated with different metal salts (equal volumes) at room temperature for 10 min. The activities were then determined under assay conditions as described above.

The kinetics parameters (K_M and V_{max}) were determined for the purified tyrosinase, using increasing tyrosine concentration as assay substrate (0.1 to 0.5 mM) at 30 °C in 100 mM phosphate buffer at pH 7.0. The K_M and V_{max} values were determined from the Michaelis-Menten plots.

3 Results and discussion

Fungi are capable of utilizing complex chemical substances as energy sources, due to their competitive saprophytic ability, which is expressed by fast mycelia growth, spores production, presence of efficient and extensive systems of powerful enzymes. In our study, a total of 25 fungi were isolated from rotting wood samples, where five (B1, B9, B13, B18, and B23) demonstrated consistent multi-enzyme characteristics by producing different zones of hydrolysis or oxidation on the screening substrates: starch, cellulose, tannic acid, pectin, casein, gum acacia, and tyrosine (Table 1). However, among these five isolates, the most consistent multi-enzyme activity was observed with isolates B1 and B13, other interesting observations were recorded in Table 1. The remarkable multi-enzyme capabilities of the fungal strains could, in part, be stimulated by the components of the medium, particularly the presence of a basic nutrient which

serves as a source of energy, and also induce the meticulous choice of metabolic pathways for nutrient breakdown, and absorption by the fungi. A similar observation of multi-enzyme capabilities was reported by Liu et al. [1], where they employed switchgrass as a source of energy and thereafter were able to achieve its saccharification through the secretion of cellulase and a retinue of accessory enzymes. Isolates B1 and B13 were identified by physical morphology and microscopy as *Fumago* sp. B13 and *Fusarium solani* B1, according to the descriptions of Pictorial Atlas of Soil and Seed Fungi [17]. The aerial white cottony mycelia on PDA, and the corresponding distinct sickle shape of the ascus suggested isolate B1 belonged to the genus, *Fusarium*. On the other hand, dark grey velvety growth on PDA, and the subsequent appearance of irregular spores borne on short conidiophores and on ordinary hyphae rich in oil globules

Table 1 Screening of fungal strains possessing enzyme activities at 72 h.

Code	Amylase	Cellulase	Pectinase	Tannase	Mannanase	Protease	Tyrosinase
(mm Zone of hydrolysis or oxidation)							
B1	23	15	19	29	16	20	18
B2	4	0	5	12	0	2	7
B3	0	13	8	0	7	5	0
B4	14	6	0	5	6	18	0
B5	12	8	12	13	0	10	7
B6	0	13	16	0	12	14	9
B7	10	0	15	11	18	0	5
B8	0	16	0	10	0	17	0
B9	17	13	15	16	11	9	10
B10	13	0	10.5	0	10	2	18
B11	15.5	20	0	6	14	5	0
B12	5	11	9	0	0	3	8
B13	18	25	17	28	17	15	20
B14	17	12	5	7.5	4.5	3	0
B15	5	6	0	8	18	7	2
B16	10	20	13.1	9	7	0	9
B17	15	7	8	9	0	0	8.5
B18	7	16	26	18	10	4	6
B19	24	6	7	4.5	6	7	5
B20	3	9	0	7	0	1	2
B21	7	2.5	0	12	8	3	1
B22	3	5	8	14	6	1	0
B23	3	15.5	17	16	12	4	6
B24	11	0	8	5	0	3	4
B25	4.5	5	0	7	7.5	8	0

were characteristic of *Fumago* sp. B13. Our findings of the *Fusarium*, an ascomycete, were congruent with the outcome of the phylogenetic study of Halaouli et al. [22], that fungal tyrosinases are clustered in basidiomycetes, ascomycetes and deuteromycetes.

3.1 Optimization studies on tyrosinase production

Enzymatic oxidation of tyrosine was carried out with the tyrosinase produced by *Fusarium solani* B1 and *Fumago* sp. B13 using atmospheric molecular oxygen, which effected the production of a black pigment [21]. In our study, it was necessary that copious enzyme titers were produced, therefore, we investigated effect of selected cultural and nutritional parameters on tyrosinase outputs by the fungi.

Maximum tyrosinase production was observed at pH of 6 and 7 in the basal medium for *Fumago* sp. B13 and *Fusarium solani* B1 respectively (Fig. 1). Tyrosinase activity ranged from 120–580 U/mL for *Fusarium solani* B1 and 100–440 U/mL in *Fumago* sp. B13 respectively. A growth medium of acidic to neutral increasingly favoured tyrosinase activity in *Fusarium solani* B1. The same trend was observed in *Fumago* sp. B13 but at pH beyond 6, activities reduced and the least activity was recorded at pH 9 (100 U/mL). One amongst other possible reasons for this phenomenon might be the inhibitory effect of the higher concentration of OH^- on fungal oxidases as pH advanced to towards neutrality, and eventually, the alkaline regime. However, Valipour and Arikan [23] reported a maximum yield of tyrosinase production between pH 6 and 7, which corroborates our observations. However, this contrasts the studies of Krishnaveni et al. [24], who opined that the optimum pH for the growth of their fungus (5.0–5.5) was in concordance with its maximum tyrosinase production.

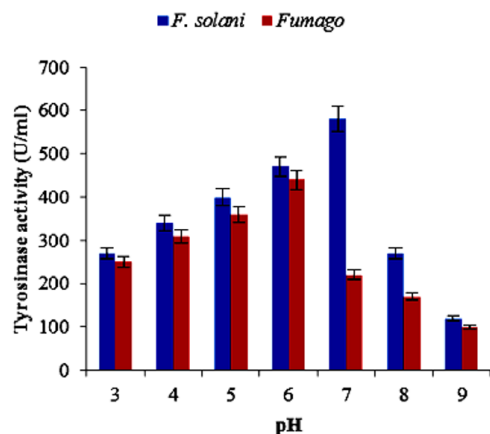


Fig. 1 Influence of pH on the production of tyrosinase by *F. solani* B1 and *Fumago* sp. B13

Glucose, when employed as the carbon source, elicited maximum tyrosinase yield in both isolates (*Fusarium solani* B1; 500 U/mL, *Fumago* sp. B13; 580 U/mL). Lactose also yielded considerably high tyrosinase titers, while a sucrose-supplemented medium produced the least tyrosinase (Fig. 2).

Agarwal et al. [25] reported the constitutive and enhanced production of tyrosinase at 2 % concentration of glucose. However, Moshtaghioun et al. [26] selected sucrose for the optimization of growth of a tyrosinase producing fungus, *Neurospora crassa* and based their preference on the presence of invertase in its membrane. As reflected in Fig. 3, maximum tyrosinase production was observed when sodium nitrate was used as the nitrogen source with an activity of 660 U/mL and 490 U/mL for *Fusarium solani* B1 and *Fumago* sp. B13. However, the least activity was observed when urea and peptone were

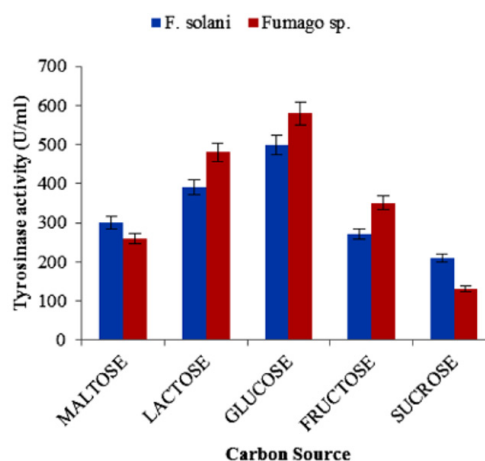


Fig. 2 Influence of different carbon sources on the production of tyrosinase by *F. solani* B1 and *Fumago* sp. B13

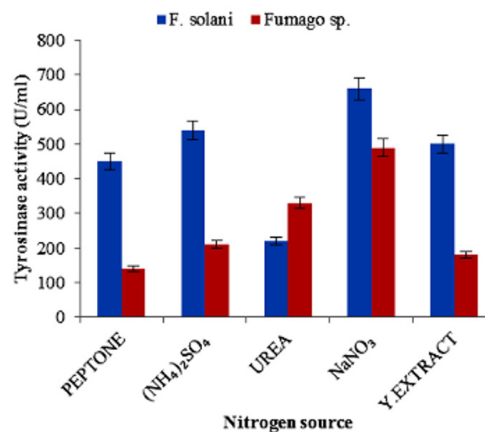


Fig. 3 Influence of different nitrogen sources on the production of tyrosinase by *F. solani* B1 and *Fumago* sp. B13

used as the nitrogen sources in the medium for *Fusarium solani* B1 (220 U/mL) and *Fumago* sp. B13 (140 U/mL). This could be due to the repressive effects of amino acids and analogues which might be present in the production of tyrosinase by some fungi [27]. Conversely, Majidi and Aksoz [28], reported that maximum enzyme production was obtained when sodium nitrate was used as nitrogen source, but they concluded that best nitrogen sources might differ from one fungus to another.

The fungal secretion of tyrosinase and its associated melanin synthesis were evaluated by the afore-mentioned exogenous sources. Fig. 4 portrayed the time course for maximum production of tyrosinase by *Fusarium solani* B1 and *Fumago* sp. B13). It was observed that tyrosinase production peaked on the 6th day (560 U/mL) and 4th day (500 U/mL) by *Fusarium solani* B1 and *Fumago* sp. B13, respectively. The factors affecting our findings may not be precisely identifiable at the moment, however, our deduction disagrees with the observations of Duarte et al. [29] and Valipour and Arikan [23], who reported 48 h and 16 h optima for *Pycnoporus sanguineus* and *Bacillus megaterium*, respectively.

3.2 Enzyme purification and characterization

The crude enzyme from *Fusarium solani* B1 and *Fumago* sp. B13 was partially purified using the ammonium sulphate fractionation (40 %) and dialysis. With each purification step, the residual tyrosinase activity of *Fusarium solani* and *Fumago* sp. reduced with the activity in dialysed samples being least (140 U/mL and 160 U/mL, respectively for *Fusarium solani* and *Fumago* sp.). However, the specific activity of the filtrates increased, hence portraying the inverse relationship between enzyme activity and the specific activity of the tyrosinases (Table 2).

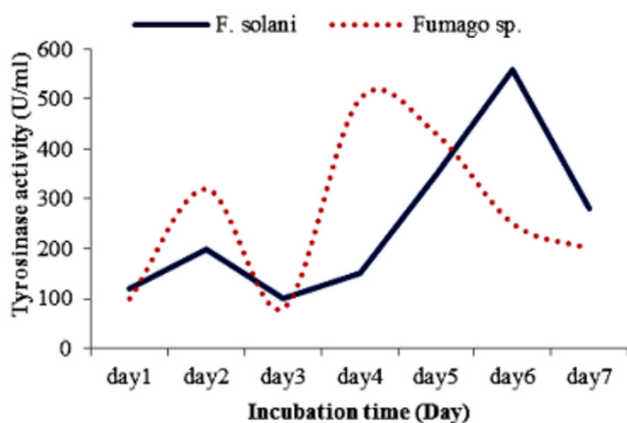


Fig. 4 Time course for the production of tyrosinase by *Fusarium solani* B1 and *Fumago* sp. B13

Fusarium solani B1 and *Fumago* sp. B13 tyrosinases were greatly enhanced by 1 mM Cu^{2+} and Mg^{2+} while other metals had little effect on the tyrosinase activities. The catalytic outputs of the tyrosinases were reduced by Hg^{2+} (Fig. 5). In a similar study, Harir et al. [11] reported the enhancing effects of preincubation of tyrosinase with Cu^{2+} and Mg^{2+} among other cations, which had little adverse effects overall.

Fig. 6 showed that *Fusarium solani* B1 and *Fumago* sp. B13 had the highest tyrosinase activity at 30 °C and the lowest at 70 °C. The increasing temperature was observed to result in a reduction of the enzyme activity, and this might be attributed to the influence the temperature of the environment wherein the fungi were isolated had on their physiology. Earlier studies have shown temperature maxima for fungal tyrosinases to range between 25 °C and 35 °C [23, 30]. Generally, it could be assumed that the susceptibility of the tyrosinases to inactivation at higher temperatures might be a result of their molecular structure, however, further studies should be conducted to ascertain this claim. Consequently, their efficiencies in applications requiring higher temperature regimes might be inconsequential. Thus, more strategic

Table 2 Purification of tyrosinase from *Fusarium solani* B1 and *Fumago* sp. B13

		Enzyme activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg protein/ml)
<i>Fusarium solani</i> B1	Crude	180	4.842598	37.17013
	$(\text{NH}_4)_2\text{SO}_4$ precipitation	170	3.455965	49.19031
	Dialysis	140	2.531543	55.30224
<i>Fumago</i> sp. B13	Crude	200	4.02436	49.69734
	$(\text{NH}_4)_2\text{SO}_4$ precipitation	190	3.106184	61.1683
	Dialysis	160	2.512804	63.67389

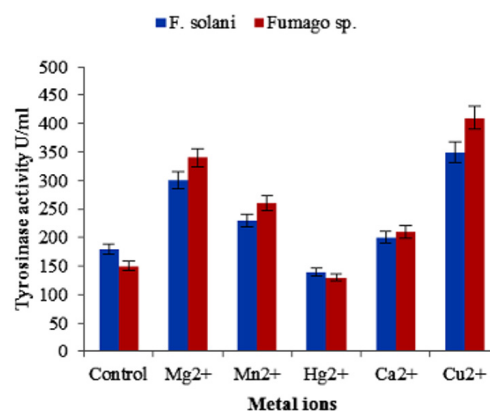


Fig. 5 Effect of metal ions (1 mM) on the tyrosinase activities of *Fusarium solani* B1 and *Fumago* sp. B13.

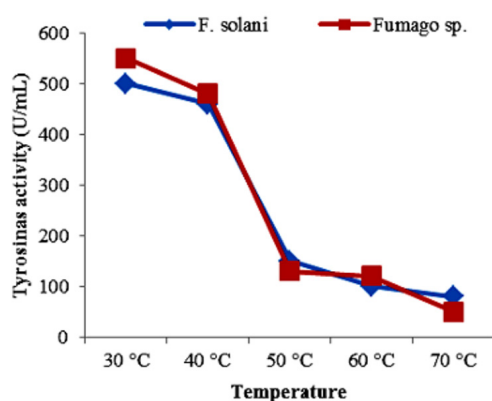


Fig. 6 Effect of temperature on the tyrosinase activities of *Fusarium solani* B1 and *Fumago sp.* B13.

screening techniques, extreme environments and structural modifications might also be considered when searching for tyrosinases with novel properties.

Fusarium solani B1 had the highest tyrosinase activity at a pH of 7 from which it gradually declined into an alkaline regime while *Fumago sp.* B13 had the highest tyrosinase activity at a pH of 6 after which it also gradually declined (Fig. 7). A possible reason for this has been earlier mentioned in the text, and our observations are in tandem with the reports of Axambayeva et al. [31] and Zou et al. [32]. Conversely, due to the harsh environments which are anticipated for real life applications, it would be highly necessary to address the bottleneck a narrow pH range would confer on catalytic activities.

It was observed that substrate concentrations (0.1–0.5 mM) was directly proportional to tyrosinase activity, where a maximum activity of 560 U/mL recorded by *F. solani* tyrosinase (Fig. 8). This is similar to the observation of Axambayeva et al. [31]. Therefore, the enzyme kinetics were extrapolated from Michaelis Menten plots using the non-linear regression method [33] where V_{max} was 0.4034 units and K_M was 0.0109 mM for *Fusarium*

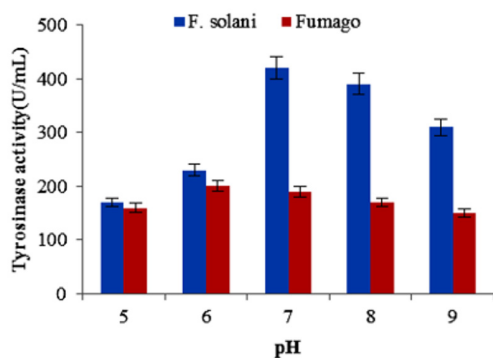


Fig. 7 Effect of pH on the tyrosinase activities of *Fusarium solani* B1 and *Fumago sp.* B13.

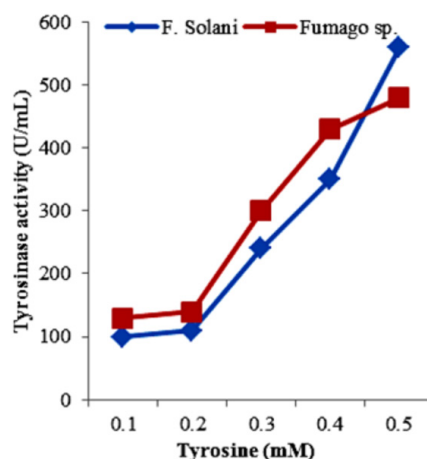


Fig. 8 Effect of increasing substrate concentration on the tyrosinase activities of *Fusarium solani* (B1) and *Fumago sp.* (B13).

solani B1 tyrosinase, and 0.4026 (V_{max}) and 0.0097 mM (K_M) for *Fumago sp.* B13 tyrosinase, respectively (Fig. 9). From the kinetics determined, it could be assumed that the fungal proteins displayed a high affinity for the substrate and could transform it at a rapid rate. Furthermore, the particular uniqueness of the tyrosinases reported in this study is their relatively higher substrate affinity, compared to other sources [11, 32, 34–36], which might imply their potential precision and efficient conversion of substrates, if applied at industrial scale. Therefore, further investigations should be encouraged to fully elucidate the potentials of these reported strains.

4 Conclusion

Fusarium solani B1 and *Fumago sp.* B13 were isolated from rotting wood samples. They were able to display remarkable zones of hydrolysis or oxidation on media supplemented with starch, cellulose, tannic acid, pectin, gum acacia, casein and tyrosine, thereby ascertaining their multi-enzyme activities and their potentials for multi-enzyme related biotechnological applications.

Tyrosinase production in these fungi was enhanced in a medium supplemented with 1 % glucose, 0.2 % NaNO_3 , pH of 6–7. Characterization of tyrosinase from *Fusarium solani* B1 and *Fumago sp.* B13 showed that the highest activities occurred at a temperature of 30 °C, in the presence of (1 mM) Cu^{2+} , at a substrate concentration of 0.5 % and pH of 6–7.

Therefore, future investigations regarding the tyrosinase from these multi-enzyme producing species should be directed at genetic manipulations and directed evolution, which would palliate the industrial production of tyrosinase from these species.

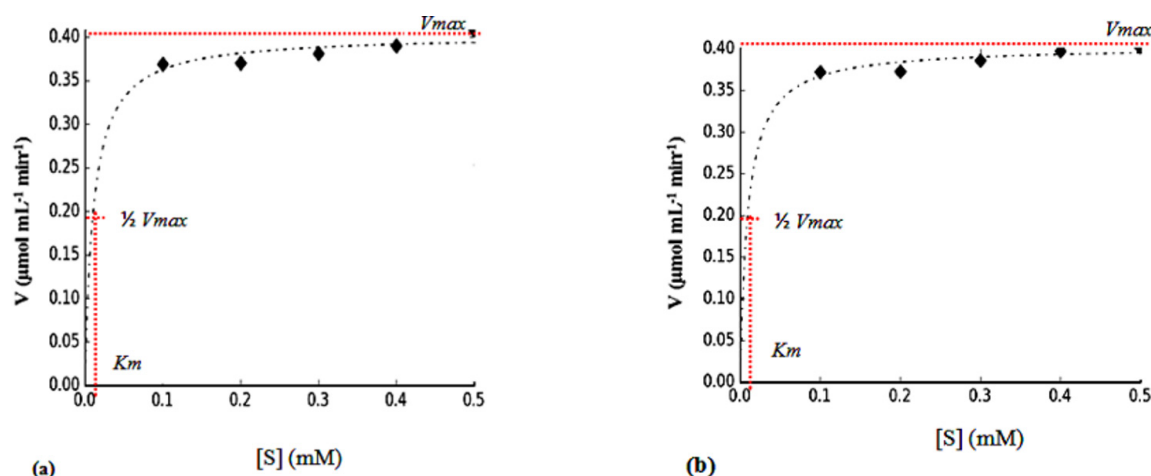


Fig. 9 Michaelis-Menten plot of the enzymatic activity of (a) *Fusarium solani* B1 and (b) *Fumago* sp. B13 tyrosinase at increasing substrate concentrations.

References

- [1] Liu, J., Sidhu, S. S., Wang, M. L., Tonniss, B., Habteselassie, M., Mao, J., Huang, Q. "Evaluation of various fungal pretreatment of switchgrass for enhanced saccharification and enzyme production", *Journal of Cleaner Production*, 104, pp. 480–488, 2015. <https://doi.org/10.1016/j.jclepro.2015.04.094>
- [2] Kang, S-Y., Heo, K. G., Hong, Y-S. "Optimization of artificial curcumin biosynthesis in E.coli by randomized 5'-UTR sequences to control the multienzyme pathway", *ACS Synthetic Biology*, 7(9), pp. 2054–2062, 2018. <https://doi.org/10.1021/acssynbio.8b00198>
- [3] Qadir, F., Shariq, M., Ahmed, A., Sohail, M. "Evaluation of a yeast co-culture for cellulase and xylanase production under solid state fermentation of sugarcane bagasse using multivariate approach", *Industrial Crops and Products*, 123, pp. 407–415, 2018. <https://doi.org/10.1016/j.indcrop.2018.07.021>
- [4] France, S. P., Hepworth, L. J., Turner, N. J., Flitsch, S. L. "Constructing biocatalytic cascades: in vitro and in vivo approaches to de Novo multi-enzyme pathways", *ACS Catalysis*, 7(1), pp. 710–724, 2017. <https://doi.org/10.1021/acscatal.6b02979>
- [5] Shepherd, L. V. T., Alexander, C. J., Hackett, C. A., McRae, D., Sungurtas, J. A., Verrall, S. R., Morris, J. A., Hedley, P. E., Rockold, D., Belknap, W., Davies, H. V. "Impacts on the metabolome of down-regulating polyphenol oxidase in potato tubers", *Transgenic Research*, 24(3), pp. 447–461, 2015. <https://doi.org/10.1007/s11248-014-9850-8>
- [6] Hamann, J. N., Herzigkeit, B., Jurgeleit, R., Tuczek, F. "Small-molecule models of tyrosinase: from ligand hydroxylation to catalytic monooxygenation of external substrates", *Coordination Chemistry Reviews*, 334, pp. 54–66, 2017. <https://doi.org/10.1016/j.ccr.2016.07.009>
- [7] Cordero, R. J. B., Casadevall, A. "Functions of fungal melanin beyond virulence", *Fungal Biology Reviews*, 31(2), pp. 99–112, 2017. <https://doi.org/10.1016/j.fbr.2016.12.003>
- [8] Xu, D.-Y., Chen, J.-Y., Yang, Z. "Use of cross-linked tyrosinase aggregates as catalyst for synthesis of L-DOPA", *Biochemical Engineering Journal*, 63, pp. 88–94, 2012. <https://doi.org/10.1016/j.bej.2011.11.009>
- [9] Zaidi, K. U., Ali, A. S., Ali, S. A., Naaz, I. "Microbial tyrosinases: promising enzymes for pharmaceutical, food bioprocessing, and environmental industry", *Biochemistry Research International*, ID 854687, 2014. <https://doi.org/10.1155/2014/854687>
- [10] Fang, Y., Ramasamy, R. P. "Detection of p-ethylphenol, a major plant volatile organic compound, by tyrosinase-based electrochemical biosensor", *ECS Journal Solid State Science and Technology*, 5(8), pp. M3054–M3059, 2016. <https://doi.org/10.1149/2.0101608jss>
- [11] Harir, M., Bellahcene, M., Baratto, M. C., Pollini, S., Rossolini, G. M., Trbalzini, L., Fatarella, E., Pogni, R. "Isolation and characterization of a novel tyrosinase produced by sahara soil actinobacteria and immobilization on nylon nanofiber membranes", *Journal of Biotechnology*, 265, pp. 54–64, 2018. <https://doi.org/10.1016/j.jbiotec.2017.11.004>
- [12] Islam, F., Roy, N. "Screening, purification and characterization of cellulase from cellulase producing bacteria in molasses", *BMC Research Notes*, 11, pp. 445, 2018. <https://doi.org/10.1186/s13104-018-3558-4>
- [13] Pinto, G. A. S., Leite, S. G. F., Terzi, S. C., Couri, S. "Selection of tannase-producing *Aspergillus niger* strains", *Brazilian Journal of Microbiology*, 32(1), pp. 24–26, 2001. <https://doi.org/10.1590/S1517-83822001000100006>
- [14] Polley, T., Ghosh, U. "Isolation and identification of potent alkaline protease producing microorganism and optimization of bio-synthesis of the enzyme using RSM", *Indian Chemical Engineer*, 60(3), pp. 285–296, 2018. <https://doi.org/10.1080/00194506.2017.1376600>

- [15] Raval, K. M., Vaswani, P. S., Majumder, D. R. "Biotransformation of a single amino acid L tyrosine into a bioactive molecule LDOPA", International Journal of Scientific Research, 2(5), pp. 1–9, 2012. Available at: <https://pdfs.semanticscholar.org/3e1f/e8f7859335a9db863f832232cf8e8afb8630.pdf> [Accessed: 25 November 2018]
- [16] Benson, H. J. "Microbiological Applications Lab Manual", 8th ed., McGraw Hill Education Publishing Company, New York, USA, 2001.
- [17] Wanatabe, T. "Identification of Fungi", In: Pictorial Atlas of Soil and Seed Fungi, Morphologies of Cultured Fungi and Key to Species. 3rd ed., CRC Press., Boca Raton, USA, pp. 9–11, 2010. <https://doi.org/10.1201/EBK1439804193>
- [18] Roy, S., Das, I., Munjal, M., Karthik, L., Kumar, G., Kumar, S., Rao, K. V. B. "Isolation and characterization of tyrosinase produced by marine Actinobacteria and its application in the removal of phenol from aqueous environment", Frontiers in Biology, 9(4), pp. 306–316, 2014. <https://doi.org/10.1007/s11515-014-1324-0>
- [19] Naraoka, T., Uchisawa, H., Mori, H., Matsue, H., Chiba, S., Kimura, A. "Purification, characterization and molecular cloning of tyrosinase from the cephalopod mollusc, *Illex argentine*", European Journal of Biochemistry banner, 270(19), pp. 4026–4038, 2003. <https://doi.org/10.1046/j.1432-1033.2003.03795.x>
- [20] Bradford, M. M. "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding", Analytical Biochemistry, 72(1–2), pp. 248–254, 1976. [https://doi.org/10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3)
- [21] Balakrishnan, G. S., Kalirajan, J. . "Identification of tyrosinase from *Dioscorea alata* tuber extract by homology driven proteomics approach", Current Proteomics, 13(1), pp. 1–8, 2016. <https://doi.org/10.2174/1570164613666160407120320>
- [22] Halaoui, S., Asther, M., Sigoillot, J.-C., Hamdi, M., Lomascolo, A. "Fungal tyrosinases: New prospects in molecular characteristics, bioengineering and biotechnological applications", Journal of Applied Microbiology, 100(2), pp. 219–232, 2006. <https://doi.org/10.1111/j.1365-2672.2006.02866.x>
- [23] Valipour, E., Arian, B. "Increased production of tyrosinase from *Bacillus megaterium* strain M36 by the response surface method", Archives of Biological Science, 68(3), pp. 659–668, 2016. <https://doi.org/10.2298/ABS151002058V>
- [24] Krishnaveni, R., Rathod, V., Thakur, M. S., Neelgund, Y. F. "Transformation of L-tyrosine to L-dopa by a novel fungus, *Acremonium rutilum*, under submerged fermentation", Current Microbiology, 58(2), pp. 122–128, 2009. <https://doi.org/10.1007/s00284-008-9287-5>
- [25] Agarwal, P., Dubey, S., Singh, M., Singh, R.P. "*Aspergillus niger* PA2 tyrosinase covalently immobilized on a novel eco-friendly bio-composite of chitosan-gelatin and its evaluation for L-DOPA production", Frontiers in Microbiology, 7, pp. 2088, 2016. <https://doi.org/10.3389/fmicb.2016.02088>
- [26] Moshtaghion, S. M., Dadkhah, M., Bahremandjo, K., Haghbeen, K., Aminzadeh, S., Legge, R. L. "Optimization of simultaneous production of tyrosinase and laccase by *Neurospora crassa*", Biocatalysis and Biotransformation, 35(1), pp. 1–10, 2017. <https://doi.org/10.1080/10242422.2016.1266617>
- [27] Faccio, G., Kruus, K., Saloheimo, Thöny-Meyer, L., "Bacterial tyrosinases and their applications", Process Biochemistry, 47(12), pp. 1749–1760, 2012. <https://doi.org/10.1016/j.procbio.2012.08.018>
- [28] Majidi, D., Aksöz, N. "Stability of tyrosinase enzyme from *Funalia trogii*", American Journal of Microbiological Research, 1(1), pp. 1–3, 2013. <https://doi.org/10.12691/ajmr-1-1-1>
- [29] Duarte, L. T., Tiba, J. B., Santiago, M. F., Garcia, T. A., Bara, M. T. F. "Production and characterization of tyrosinase activity in *Pycnoporus sanguineus* CCT-4518 crude extract", Brazilian Journal of Microbiology, 43(1), pp. 21–29, 2012. <https://doi.org/10.1590/S1517-83822012000100003>
- [30] Bayranoglu, G., Akbulut, A., Arica, M. Y. "Immobilization of tyrosinase on modified diatom biosilica: enzymatic removal of phenolic compounds from aqueous solution", Journal of Hazardous Materials, 244–245, pp. 528–536, 2013. <https://doi.org/10.1016/j.jhazmat.2012.10.041>
- [31] Axambayeva, A. S., Zhaparova, L. R., Shagyrova, Zh. S., Ramankulov, E. M., Shustov, A. V. "Unusual stability of a recombinant *Verrucomicrobium spinosum* tyrosinase to denaturing agents and its use for a production of a protein with adhesive properties", Applied Biochemistry and Biotechnology, 185(3), pp. 736–754, 2018. <https://doi.org/10.1007/s12010-017-2686-y>
- [32] Zou, Y., Hu, W., Jiang, A., Ma, K. "Partial purification and characterization of a novel extracellular tyrosinase from *Auricularia auricula*", Applied Biochemistry and Biotechnology, 172(3), pp. 1460–1469, 2014. <https://doi.org/10.1007/s12010-013-0638-8>
- [33] Marasović, M., Marasović, T., Milos, M. "Robust nonlinear regression in enzyme kinetics parameters estimation", Journal of Chemistry, 2017, ID: 6560983, 2017. <https://doi.org/10.1155/2017/6560983>
- [34] Pretzler, M., Bijelic, A., Rompel, A. "Heterologous expression and characterization of functional mushroom tyrosinase (AbPPO4)", Scientific Reports, 7(1), pp. 1810, 2017. <https://doi.org/10.1038/s41598-017-01813-1>
- [35] Wan, X., Chai, B., Liao, Y., Su, Y., Ye, T., Shen, P., Chen, X. "Molecular and biochemical characterization of a distinct tyrosinase involved in melanin production from *Aeromonas media*", Applied Microbiology and Biotechnology, 82, pp. 261–269, 2008. <https://doi.org/10.1007/s00253-008-1742-5>
- [36] Selinheimo, E., Saloheimo, M., Ahola, E., Westerholm-Parvinen, A., Kalkkinen, N., Buchert, J., Kruus, K. "Production and characterization of a secreted, C-terminally processed tyrosinase from the filamentous fungus *Trichoderma reesei*", The FEBS Journal, 273(18), pp. 4322–4335, 2006. <https://doi.org/10.1111/j.1742-4658.2006.05429.x>